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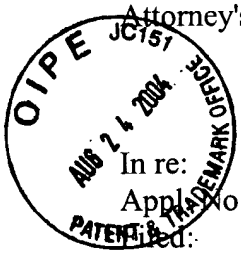
Attorney's Docket No. 044158/209598 PATENT



In The United States Patent And Trademark Office

In re:	Evans et al.	Confirmation No.:	2302
Appl. No.:	09/829,113	Group Art Unit:	1637
Filed:	April 9, 2001	Examiner:	Jeffrey Norman Fredman
For:	HAPLOTYPING METHOD FOR MULTIPLE DISTAL NUCLEOTIDE POLYMORPHISMS		

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APPEAL BRIEF TRANSMITTAL
(PATENT APPLICATION – 37 C.F.R. § 1.192)

1. Transmitted herewith, in **triplicate**, is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on August 5, 2004.

2. ☒ Applicant claims small entity status.

3. Pursuant to 37 C.F.R. § 1.17(c), the fee for filing the Appeal Brief is:
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Attorney's Docket No. 044158/209598 PATENT

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APPEAL BRIEF UNDER 37 CFR § 1.192

This Appeal Brief is filed pursuant to the "Notice of Appeal to the Board of Patent Appeals and Interferences" filed 8/5/2004.

1. ***Real Party in Interest.***

The real party in interest in this appeal is St. Jude Children's Research Hospital, Inc., the assignee of the above-referenced patent application.

2. ***Related Appeals and Interferences.***

There are no related appeals and/or interferences involving this application or its subject matter.

3. ***Status of Claims.***

Claims 1-18 and 21-22 are the subject of this appeal. The claims appear in **Appendix A**.

4. **Status of Amendments.**

No amendments have been made subsequent to the final rejection.

5. ***Summary of the Invention.***

Generally, Applicants disclose a method for determining haplotype structure. See claims 1-18 and 21-22. Reliable and quick methods for determining haplotype structure are important because these structures are frequently the principal determinant of differences in disease risk and treatment response (drug metabolism, etc.) between individual humans. See the specification, page 1, lines 12-22.

By way of explanation, a haplotype structure is composed of nucleotide polymorphisms. A nucleotide polymorphism is a known variation in a DNA sequence at a particular location among contiguous DNA segments that are otherwise similar in sequence (for example, a gene or any other portion of a chromosome); the haplotype is the particular nucleotide sequence of the nucleotide polymorphism. See the specification, page 1, lines 12-22. The combination of haplotypes present on a contiguous DNA segment or an allele of a gene or other portion of a chromosome structure is the haplotype structure. Prior to Applicants' disclosure, methods for determining haplotype structure were limited to haplotypes separated by short stretches of DNA, were limited to particular forms of polymorphisms, or were complicated.

Applicants disclose and claim a method for determining the haplotype structure of a contiguous DNA segment having at least two nucleotide polymorphisms separated by at least 200 nucleotides. The method comprises the following steps:

(1) Obtaining a DNA sample comprising the contiguous DNA segment. See claim 1 and the top panel of **Appendix B.** (The figure is a modified version of Figure 1 from the application, which depicts Applicants' proof-of-concept work. It has been modified by removal of the final panel to emphasize that any method of haplotype determination can be used.)

(2) Using the DNA sample as a template for polymerase chain reaction (PCR) amplification of a DNA fragment comprising the DNA segment. See claim 1 and the second panel of **Appendix B**.

(3) Ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule, wherein the first nucleotide polymorphism and the second nucleotide polymorphism are brought into closer proximity on the circular DNA molecule relative to the contiguous DNA segment. See claim 1 and the third panel of **Appendix B**.

(4) Determining the haplotype of the first nucleotide polymorphism and the second nucleotide polymorphism. See claim 1. Although Figure 1 of the application depicts the fourth step carried out via a second round of PCR, methods for determining haplotype that can be used with Applicants' invention include standard art-recognized techniques including restriction fragment-length polymorphism (RFLP) analysis, single-strand conformational polymorphism analysis, heteroduplex analysis, oligonucleotide ligation, hybridization assays, PCR-RFLP, allele-specific amplification, single-molecule dilution, coupled amplification and sequencing, and the like. See the specification, page 4, lines 8-12[1] and **Appendix B.**

Applicants' disclosed technique can be used with nucleotide polymorphisms separated by at least 10,000, 20,000, or 30,000 nucleotides and is compatible with long-range PCR. See claims 2-4 and 10. By bringing the nucleotide polymorphisms into closer proximity through circularization, analysis of haplotype structure can be achieved by methods known in the art that could not previously be employed in determining the haplotype structure of DNA segments and genes with distantly spaced nucleotide polymorphisms. See the specification, page 3, lines 21-24. Applicants' technique can be used with more than two nucleotide polymorphisms of various forms present in the genome of organisms including *Homo sapiens*. See claims 5-6 and 14-18.

Using PCR to isolate the DNA fragment containing the target nucleotide polymorphisms prior to circularization is advantageous because a scientist following Applicants' invention can control both the number of polymorphisms on the fragment and the distance between the nucleotide polymorphisms on the circular molecule by designing PCR primers that will bind a given distance away from the nucleotide polymorphism. See the top two panels of **Appendix B**; claims 7-9 and 21-22; and the specification, page 7, lines 6-14. Thus, the distance between the polymorphisms can be optimized for whatever technique of haplotype determination the scientist wishes to utilize with Applicants' invention.

The use of PCR to generate the target DNA fragment prior to circularization provides further advantages, including the ability to generate substantial quantities of target DNA to ensure robust ligation and circularization, as well as the option to design particular "sticky ends" for the DNA fragment. See claim 11 and the specification, page 7, lines 15-30. Applicants'

[1]Figure 1 sets forth in schematic fashion one embodiment of Applicants' invention in which long range PCR is utilized to generate the DNA fragment.

invention is also easy to perform and requires instrumentation and reagents that are widely available. See the specification, page 18, lines 26-27.

6. ***Issues.***

Issue 1—Whether the subject matter of Claims 1-18 and 21-22 are nonobvious under 35 U.S.C. § 103.

7. ***Grouping of Claims.***

The claims do not stand or fall together. Claims 1-18 and 21-22 are pending. Claims 1-16 and 21-22 have been rejected under 35 U.S.C. § 103 based on the Examiner's conclusion that it would be obvious to combine a reference demonstrating isolation of a DNA fragment containing nucleotide polymorphisms separated by 30-43 base pairs by PCR, ligating the PCR fragment, and carrying out allele-specific inverse PCR with (1) a reference disclosing allele-specific long-range PCR and (2) a reference disclosing carrying out restriction digestion of an organisms entire genome, ligating the restriction fragments, and carrying out inverse PCR to simultaneously isolate and haplotype a particular region of genomic DNA. The Examiner has rejected each of claims 17 and 18 separately. However, for purposes of appeal, claims 12 and 22 should also be considered separately, as explained in the following paragraphs.

Claim 12 is drawn to an embodiment in which the haplotype of the polymorphisms on the circular DNA molecule is detected by restriction fragment analysis of the circularized segment. No reference is cited for teaching or suggesting this limitation and claim 12 should be considered separately.

Claim 17 is subject to rejection over an additional secondary reference to Krynetski *et al.* (1995) *Proc. Natl. Acad. Sci.*, 92:949-953. No motivation has been established for the combination of Li *et al.* with Patel *et al.* and Michalatos-Beloin *et al.*, nor is any provided for the addition of the Krynetski *et al.* reference. Further, the haplotype structure referred to in claim 17 is comprised of nucleotide polymorphisms that are roughly 8 kilobases apart. It is Applicants' proof of concept work that teaches the method and likelihood of success of the method to the haplotype structure. Because of these additional issues, claim 17 should be considered separately.

Claim 18 is subject to rejection over an additional secondary reference to Martin *et al.* (2000) *Am. J. Hum. Genet.* 67:383-394. For reasons of record, there is no motivation to combine Li *et al.* with Patel *et al.* and Michalatos-Beloin *et al.* Martin *et al.* teaches SNPs in the region surrounding the APOE gene but does not satisfy the deficiencies of the primary references. Accordingly, claim 18 should be considered separately.

Claim 22 specifies that the DNA sequence immediately 5' to the first nucleotide polymorphism and the DNA sequence immediately 3' to the second nucleotide polymorphism have lengths selected from less than 500, less than 400, less than 300, less than 200, less than 100, or less than 50 bases long, respectively. None of the references teach or suggest such a selection step. Thus, claim 22 should be considered separately.

8. ***Argument.***

In the present case, the Examiner has failed to establish by objective evidence the required motivation to combine the references cited in the rejection. In past responses, Applicants explained that the rejection relies upon Applicants' own teaching to provide the motivation for the combination of references asserted. In reply, the Examiner asserts that Applicants are improperly arguing against the references individually instead of as a whole. However, the references should not be taken as a whole because the Examiner has not established the motivation for their combination by objective evidence. Applicants also submitted a Rule 132 Declaration in support of their contention that the references fail to provide the motivation for their combination. The Examiner dismissed this affidavit as incorrect, but has failed to show by evidence how the statements of the affidavit are erroneous. Further, even if the references are combined, they fail to produce Applicants' invention. The record fails to describe the modification of each reference in a way that produces Applicants' invention and, as a consequence, never reaches the issue of whether a basis for the motivation for each modification exists.

For all of these reasons, the rejections should be reversed.

A. The Rejection of Claims 1-11, and 13-16 Cannot be Upheld.

Claims 1-11 and 13-16 have been rejected by the Examiner under 35 U.S.C. § 103 over Li *et al.* (1998) *BioTechniques* 25:358-361 in view of Patel *et al.* (1991) *Nucleic Acids Res.*, 19:3561-3567 and Michalatos-Beloin *et al.* (1996) *Nucleic Acids Res.* 24:4841-4843.

It is the examiner's burden to present a prima facie case of obviousness by establishing that the teachings of the prior art itself would appear to have suggested the claimed subject matter to a person of ordinary skill in the art. *In re Rejckaert*, 9 F.3d 1531, 28 U.S.P.Q.2d 1955 (Fed. Cir. 1993). In particular, "[t]o prevent the use of hindsight based on the invention to defeat patentability of the invention, [the Federal Circuit] requires the examiner to show a motivation to combine the references that create the case of obviousness." *In re Rouffet*, 149 F.3d 1350, 47 U.S.P.Q.2d 1453 (Fed. Cir. 1998). A demonstration of motive to combine must be based on objective evidence of record. *In re Lee*, 277 F.3d 1338, 61 U.S.P.Q.2d 1430, 1433 (Fed. Cir. 2002). Accordingly, the Examiner bears the burden of establishing by objective evidence the motivation to combine the references and, if this burden is not fulfilled, the references as a whole do not teach or suggest the invention.

As discussed, Applicants' method for determining the haplotype structure of a contiguous DNA segment comprising at least two nucleotide polymorphisms comprises the steps of obtaining a DNA sample for use as a template for PCR[2] amplification of a DNA fragment;

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[2] For the convenience of the reader unfamiliar with the PCR method, Applicants' include the following short explanation:

> So simple is the PCR process, at least to molecular biologists, that its inventor, Kary Mullis, says their universal reaction has always been, "Why didn't I think of that?"
>
> ....
>
> PCR requires a template molecule-the DNA or RNA you want to copy-and two primer molecules to get the copying process started.
>
> ....
>
> For PCR, primers must be duplicates of nucleotide sequences on either side of the piece of DNA of interest, which means that the exact order of the primers' nucleotides must already be known.
>
> ....
>
> There are three basic steps in PCR. First, the target genetic material must be denatured...and separated-by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand.
>
> ....

amplifying the fragment; ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule, thereby bringing the nucleotide polymorphisms into closer proximity on the circular DNA molecule relative to the contiguous DNA segment; and determining the haplotype of the nucleotide polymorphisms. The references as a whole do not teach or suggest the claimed invention nor does the objective evidence support their combination. Instead, the Examiner has improperly relied on hindsight to select and combine the references. This is particularly evident in the Examiner's conclusions regarding Li *et al.*

The Examiner asserts that Li *et al.* teaches Applicants' second and third steps; i.e., producing a PCR fragment containing more than one polymorphism, then ligating the fragment to circularize the product and bring the polymorphisms into closer proximity on the circularized product. To the contrary, although Li *et al.* circularizes a PCR fragment containing more than one polymorphism, the polymorphisms were roughly 30-43 base pairs before circularization and are no closer than this after circularization. Figure 1 of Li *et al.* clearly demonstrates this point. For the reader's convenience, a copy of Li *et al.* has been included as **Appendix C**. Consequently, Li *et al.* fail to teach or suggest closer proximity of the polymorphisms resulting from performance of Applicants' second and third steps.

The Examiner also asserts that Li *et al.* discuss applying their technique to widely spaced polymorphisms and that one of skill in the art would be induced to use long-range PCR to solve this problem. However, the problem faced by Li *et al.* was not amenable to using long-range PCR to produce a DNA fragment for circularization. Rather, the problem identified by Li *et al.* was caused by the inability to design a single allele-specific primer due to the roughly 30-43 base pairs between the polymorphisms. Applicants have brought the portion of Li *et al.* that discusses the primer design problem to the Examiner's attention. However, the Examiner has dismissed this objective evidence in favor of his subjective interpretation that Li *et al.* was discussing a

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> To get more of the DNA you want, just repeat the process, beginning by denaturing the DNA you've already made. The amount will double every time.

The Polymerase Chain Reaction, Tabitha M. Powledge, Federation of American Societies for Experimental Biology, http://www.pcrlinks.com/generalities/introduction.htm.

problem amenable to long-range PCR. This subjective interpretation only arises when Li *et al.* is viewed with hindsight in light of the Applicants' disclosure. This is improper.

The Examiner cites Michalatos-Beloin *et al.* for teaching long range PCR. However, the rejection fails to take into account Michalatos-Beloin *et al.* teaching away from its combination with other techniques (its advantage lies in the fact that it is a *single* step procedure in which the target sequence is (a) isolated and (b) haplotyped with a single PCR step).

The Examiner cites Patel *et al.* for its teaching of inverse PCR from genomic DNA. This teaching is inapplicable because Applicants' claimed method does not depend upon inverse PCR (or any particular method of determining haplotype).

Because of these deficiencies, the rejection should have been withdrawn. However, when confronted with these deficiencies the Examiner replied that Applicants improperly argued against the references individually instead of as a whole. However, controlling Federal Circuit precedent requires the Office to establish basis for the combination, and the evidence of record does not support the combination of the presently cited references.

Even if the references were combined, the Examiner fails to discuss the modifications he implies that the art worker would make and, consequently, fails to reach the issue of motivation to modify.

Applicants have also submitted a Rule 132 declaration explaining the technical teachings of each reference, a copy of which is provided for the convenience of the reader as **Appendix D**. In his reply, the Examiner argues that Applicants' declaration is wrong and "distorts" the teachings of the primary reference. However, no evidence is provided—in some cases no explanation is provided—to support these arguments. This is improper because the Examiner has the burden of providing at least some evidentiary basis for dismissing Applicants' Rule 132 evidence.

Each of these points is discussed in detail in the following sections.

1. The Examiner's Hindsight Rejection Improperly Relies Upon Applicants' Disclosure.

As set forth above, Applicants disclose and claim a method that comprises the steps of (1) obtaining a DNA sample comprising a contiguous DNA segment; (2) using the

DNA sample as a template for polymerase chain reaction (PCR) amplification of a DNA fragment comprising said contiguous DNA segment; (3) ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule wherein said first nucleotide polymorphisms and said second nucleotide polymorphisms are brought into closer proximity on said circular DNA molecule relative to said contiguous DNA segment; and (4) determining haplotype. As a proof-of-concept, Applicants used the method for determining the haplotype structure of individual human's TPMT genes. See the specification, page 16, line 3 to page 21, line 15. It is Applicants' disclosure that teaches the ease of performance and reliability of the invention.

Nonetheless, the Examiner rejects Applicants' claimed invention as obvious over Li *et al.* in view of Patel *et al.* and Michalatos-Beloin *et al.* The Examiner incorrectly asserts that the references teach or suggest the second and third steps of Applicants' invention. The teachings of each of these references is summarized below.

a. Li et al. Discloses A Method That Does Not Bring Polymorphisms Into Closer Proximity.

Li *et al.* developed a protocol for determining the haplotype structure of the MN blood group system, a relatively short haplotype structure in which the nucleotide polymorphisms are separated by less than 100 bases in genomic DNA. Their protocol was not developed to determine haplotype structures composed of widely separated polymorphisms and does not involve bringing the polymorphisms into closer proximity on the circular DNA molecule.

To place Li *et al.* in context, one must understand the MN blood type system (the focus of their publication). The MN blood type is determined by genetic polymorphisms within the GPA gene of red cells. The particular combination of haplotypes present in an individual yield three haplotype structures (termed M^G, M^T, and N, respectively). Haplotyping the MN system presents two problems. First, the sequence of the GPA gene is extremely similar (over 95% homologous to) the GPB and GPE genes. In fact, the polymorphisms that determine the M^G, M^T, and N alleles of the GPA gene also occur in the GPB and GPE genes. Thus, at least one primer has to be GPA specific. The second problem arises because the M^T - (and N-) specific

nucleotide T in intron 1 is too distant (30-40 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T -specific primer.[3] Li *et al.* take pains to describe these problems on page 358, columns 1-2 (the reference may be found in **Appendix C**).

Previous workers had applied PCR-based haplotyping techniques to the MN blood system. These previous workers had solved the homology problem (the first problem) by using at least one PCR primer that bound to sequence unique to the GPA gene. Li *et al.*, page 358, columns 1-2. Because of the distance problem (the second problem), the previous workers had utilized restriction-fragment length polymorphism or single-strand conformation polymorphism analysis, but not the allele-specific PCR-amplification technique. *Id.*

Li *et al.* developed a method by which they were able to utilize allele-specific PCR, albeit allele-specific *inverse* PCR, to solve the second problem. Like others in the field, they solved the homology problem by utilizing primers that bound to sequence unique to the GPA gene (i.e., on either side of the region containing polymorphisms that determine the M^G, M^T, and N alleles). However, Li *et al.* chose to solve the distance problem with allele-specific inverse PCR (sometimes called inside-out PCR), which involves the generation of circular molecules via ligation and can be applied to closely linked polymorphisms. Li *et al.*, paragraph spanning pages 360-1. Thus, the method of Li *et al.* involves three general steps: Generation of a short linear gene-specific PCR product from the MN region of the GPA gene; ligation of the PCR product; and performance of allele-specific inverse PCR (ASIP) using allele-specific inverse primers. See Li *et al.*, page 360, Figure 1.

In every rejection involving Li *et al.*, the Examiner asserts that the Li *et al.* method is functionally similar to Applicants' method and provides the motivation for its combination with the long-range PCR technique of Michalatos-Beloin *et al.* In particular, the Examiner maintains that Li *et al.* teaches a method of determining the haplotype structure of a contiguous DNA "wherein the ligation brings the first and second polymorphisms into closer proximity on the circular DNA molecule..." and suggests an unsolved problem, namely that "some haplotypes are too distant to be amplified by standard PCR." According to the Examiner, this unsolved problem

[3] The alleles discussed in Li *et al.* are as follows:
- M^G: G...[30-40 base pairs]...C...GT
- M^T: T...[30-40 base pairs]...C...GT
- N: T...[30-40 base pairs]...T...AG

provides the motivation for the combination of Li *et al.* with Michalatos-Beloin *et al.* and for the application of Li *et al.* to haplotypes comprised of widely separated nucleotide polymorphisms.

Contrary to the Examiner's assertions, a review of Li *et al.* reveals that their ligation technique *does not* bring polymorphisms into closer proximity on the circular DNA molecule. The polymorphisms are roughly 30-43 base pairs apart in genomic DNA. Once circularized, the closest distance between the polymorphisms remains roughly 30-43 base pairs. Moreover, Li *et al.* do not identify an unsolved problem.[4] Although Li *et al.* does identify a problem involving distance, the distance problem was the roughly 30-43 bases that separated the nucleotide polymorphisms and prevented previous art-workers from applying allele-specific PCR-amplification to haplotyping the MN blood system. Li *et al.* solve this problem using their technique.

To use Li *et al.* in the way asserted by the Examiner, the method would at least have to be modified so that the polymorphisms were brought closer by circularization and then applied to widely spaced polymorphisms. Where the examiner asserts that it would be obvious for the art worker to modify the prior art, the mere fact that the prior art may be modified in the manner suggested by the Examiner does not make the modification obvious unless the prior art suggested the desirability of the modification. *In re Fritch*, 972 F.2d 1260, 1265, 23 U.S.P.Q.2d 1780, 1783 (Fed. Cir. 1992). Here, nothing in Li *et al.* suggests the desirability of applying their technique to widely separated nucleotide polymorphisms. Further, nothing suggests the desirability of modifying the PCR scheme by altering the primer placement such that the nucleotide polymorphisms would be brought into closer proximity by circularization. Thus, Li *et al.* does not teach or suggest Applicants' claimed method and fails to provide the motivation for either the combination or modification of the references cited in the rejection.

[4]Even if Li *et al.* did identify an unsolved problem, Applicants' representative notes that the identification of an unsolved problem is an indicia of *nonobviousness*, not objective evidence of motivation to combine with another reference.

b. Patel et al. Fail to Provide the Motivation for the Combination of References Asserted by the Examiner

Patel *et al.* teaches a multi-step method of determining the haplotype of an individual. First, a restriction digest is carried out on genomic DNA isolated from an individual to be haplotyped. Second, the DNA fragments generated from the restriction digest are circularized using a DNA ligase. Third, the fragment is re-linearized utilizing a different restriction enzyme. Fourth, the haplotype is assessed by inverse PCR utilizing Amplification Refractory Mutation System primers. Patel *et al.* does not isolate the target DNA fragment comprising the nucleotide polymorphisms prior to ligation, nor do they use a PCR step prior to ligation. Thus, the technique of Patel *et al.* cannot take advantage of any of the benefits of isolating the target DNA fragment via PCR prior to ligation, such as optimizing the distance between the polymorphisms on the circular molecule to the chosen haplotyping technique, generating substantial quantities of target DNA, introducing "sticky ends" to the DNA fragment, etc. Accordingly, Patel *et al.* would not provide the motivation for its combination with any of the other references.

c. Michalatos-Beloin et al. Teaches Away from the Combination of References Asserted by the Examiner

Michalatos-Beloin *et al.* teaches a one-step method of haplotype differentiation based on the use of allele-specific primers and the differing size or presence of PCR products depending on the haplotype structure. Unlike either Li *et al.* or Patel *et al.*, no circularization of the long-range PCR products is required, nor would such circularization be of any benefit for this method. Indeed, the Michalatos-Beloin *et al.* method teaches isolation of DNA in the step of haplotyping; i.e., by a single-step PCR protocol. Michalatos-Beloin *et al.* tout the advantages of the single-step method, stating "The ability to isolate hemizygous DNA segments readily from heterozygous genomes via molecular haplotyping will provide the accuracy necessary in these diverse applications' (page 4867, column 2)." Michalatos-Beloin *et al.* teaches away from its combination with methods that require additional steps to analyze the haplotype of a sequence of interest. Li *et al.*, on the other hand, teaches a multistep PCR

protocol directed to solving the related problems of spurious product formation from homologous genes and difficulty of designing a single primer. Because Michalatos-Beloin *et al.* teaches away from its combination with methods that require additional steps to analyze the haplotype of a sequence of interest, the art worker would not be motivated to utilize Michalatos-Beloin *et al. with* Li *et al.* (or Patel *et al.*, for that matter). Rather, the art worker would be motivated to utilize Michalatos-Beloin *et al. alone.*

d. The Rejection Should Be Reversed Because The Objective Evidence Fails To Demonstrate That The Skilled Art Worker Would Be Motivated To Combine The References Cited By The Examiner.

A review of the references cited by the Examiner reveals that there is no objective evidence demonstrating that the skilled art worker would be motivated to combine them. Rather, the objective evidence contradicts the alleged teachings of Li *et al.* upon which the Examiner bases motivation to combine: As explained above, Li *et al.* does not teach or suggest a method which produces a circular DNA molecule wherein the polymorphisms are brought into closer proximity on the circular DNA molecule relative to the template DNA segment. It is Applicants' disclosure that provides this teaching. Further, Li *et al.* does not provide the motivation for its application to haplotypes comprised of widely separated nucleotide polymorphisms. It is Applicants' disclosure that provides this teaching, as well.

The technique of Patel *et al.* is dissimilar to the other references and does not involve PCR until the final step. It cannot take advantage of any of the benefits of isolating the target DNA fragment via PCR prior to ligation and fails to provide the motivation for its combination with any of the other references.

Michalatos-Beloin *et al.* teaches away from its combination with methods that require additional steps to analyze the haplotype of a sequence of interest.

For these reasons, the objective evidence does not support a conclusion that the skilled art worker would be motivated to combine the references cited in support of the rejection and the Examiner has not carried his burden in supporting the obviousness rejection. Applicants have brought these deficiencies to the attention of the Examiner. See the response mailed March 22, 2004, pages 2-11 and the response mailed October 2, 2003, pages 6-15. Applicants have even

submitted a Rule 132 Declaration explaining the teachings of the references. See the response filed March 22, 2004. In response, the Examiner has maintained the rejections, but has declined to offer evidence to correct the deficiencies of the rejection, as explained in the remainder of the brief.

2. The Examiner's Responses Have Not Cured the Deficiencies of the Rejection

Although Applicants have drawn attention to the lack of motivation to combine the references and the rejection's reliance upon hindsight analysis, the Examiner continues to accredit Applicants' own teachings to Li *et al.* Specifically, the Examiner incorrectly maintains that Li *et al.* teaches a method of determining the haplotype structure of a contiguous DNA segment by using PCR to produce a DNA fragment containing more than one polymorphism and ligating the ends of the DNA fragment to produce a circular DNA molecule "wherein the ligation brings the first and second polymorphisms into closer proximity on the circular DNA molecule...." See the Office Action mailed 5/5/2004, page 4, first line. Further, the Examiner improperly maintains his subjective conclusion that the final sentence of Li *et al.* provides the motivation for its combination with Michalatos-Beloin *et al.* These positions are unsupportable, for reasons described in detail in the following sections.

a. The Examiner's Responses Fail to Show that Li et al. Brings Polymorphisms Into Closer Proximity.

Applicants have submitted evidence in the form of a Rule 132 Declaration by the inventors explaining that the polymorphisms are no closer. The relevant paragraph is set forth below for the reader's convenience.

> Li *et al.* does not circularize in a way that brings polymorphisms closer together the way our claimed method does. Figure 1 of our application and Figure 1 of Li *et al.* clearly demonstrates this result. When in linear genomic DNA, the distal-most polymorphisms, M-G/T in intron 1 and M(G/T)-N(AG) in exon 2, are separated by 30-43 bp (page 358, second column, line 12). Once circularized by Li *et al.*, the polymorphisms are the *same* distance from each other in one direction of the circle and *farther apart* in the other direction (a 357 bp linear molecule was circularized, thus 357 minus 30-43 gives the polymorphisms a distance of between 314 to 327 bps from each other).

> Li *et al.* does not provide the advantages of our method. By using inverse PCR, Li *et al.* simply generate back the same, or nearly the same, linear molecule that was circularized by using inverse PCR (after inverse PCR off of a 357 bp circle, Li *et al.* generate a 351 and a 366 bp fragment). In my scientific opinion, had Li *et al.* intended haplotype analysis of circular molecules the way our method allows (i.e. aimed at analyzing polymorphisms that had been brought into closer proximity), then Li *et al.* would *not* have used inverse PCR because inverse PCR simply generates back the same, or nearly the same, linear molecule that was circularized in the first place.

See the Declaration of Drs. McDonald and Evans, ¶ 8, filed March 22, 2004, attached herewith for the convenience of the reader as **Appendix D.**

The Examiner has dismissed this objective evidence in favor of his subjective interpretation of Li *et al.* For instance, the Examiner responds to Applicants' evidence that Li *et al.* teaches a method that *does not* bring the relevant polymorphisms into closer proximity by stating "This is not correct." See the Office Action mailed 5/5/2004, page 9, 3rd ¶, line 2. The Examiner never points out any statement or figure in Li *et al.* that shows that the MN nucleotide polymorphisms are in closer proximity on the circular molecule. Instead, the Examiner states that the Li *et al.* "method of ASIP requires restriction digestion and ligation of circles to bring *ends* closer together." Office Action mailed 5/5/2004, page 12 (emphasis added). This does not address Applicants' point, however, because the *polymorphisms* of Li *et al.* are not at opposite ends of the contiguous DNA segment such that circularization would bring them closer together. Rather, the objective evidence shows they are 30-43 base pairs apart at one end of the contiguous DNA segment and remain 30-43 base pairs apart after circularization. See Figure 1 of Li *et al.*

The Examiner also asserts that "ASIP is functionally identical to Applicants' invention, except for the requirements that there be a separation of 200, 1000, 10,000, or 30,000 nucleotides." See the Office Action mailed 5/5/2004, last line of page 9. As with the other statements in reply to the declaration, no evidence is cited for this premise, likely because none can be found. The method is not functionally identical because Li *et al.* does *not* bring the nucleotide polymorphisms into closer proximity on the circular molecule (See Figure 1 of Li *et al.*). Li *et al.* is not applicable to haplotype structures comprising widely separated nucleotide polymorphisms.

With respect to the meaning of the final sentence of Li *et al.*, the Examiner responds to Applicants' Declaration with the following conclusory statement: "Li would not have said that the 30-40 bases between the polymorphisms was a distance too long to be amplified by PCR when he amplified a 357 base pair fragment. Consequently, Li must have been referring, in his quote, to other polymorphisms which are too far apart for PCR amplification." See the Office Action mailed 5/5/2004, page 10, first full paragraph. This is pure conjecture. Contrary to the Examiner's conclusion, Li *et al.* state that the MN alleles can be typed by restriction fragment length polymorphism or single-strand conformation polymorphism analysis, *but not by the single allele-specific PCR amplification* technique. Li *et al.*, page 358, column 1. They then take pains in column 2 to explain that this is because the "M^T- (and N-) specific nucleotide T in intron 1 is located too far (30-43 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T-specific primer." Therefore, Li *et al. do teach* that a 30-43 base pair distance prevented a form of PCR amplification.

Li *et al.* does not teach or suggest bringing the polymorphisms into closer proximity on the circular DNA molecule. Moreover, the Examiner fails to explain why the art worker would be motivated to modify Li *et al.* such that the polymorphisms were brought into closer proximity.

b. The Examiner's Responses Are Founded Upon His Subjective Construction of the Final Sentence of Li et al.

In the final sentence of the publication, Li *et al.* summarizes their work by stating that "Although these alleles can be typed by allele-specific nested PCR following GPA-specific PCR, ASIP, rather than nested PCR, can be applied to haplotyping of polymorphisms separated by a distance that is too long to be amplified by PCR." The Examiner has seized upon this sentence to conclude that "An ordinary practitioner would be therefore be motivated to use long range PCR to prepare the template for the method of Li in order to extend the range of detection of polymorphisms in order to solve the problem of Li that there are 'polymorphisms separated by a distance that is too long to be amplified by PCR'." See the Office Action mailed 5/5/2004, page 6. The Examiner then asserts that "Michalatos-Beloin solves the problem using long range PCR." *Id.*

As evidenced by these quoted portions of the Office Action, the Examiner has read the final sentence of Li *et al.* in light of Applicants' disclosure to intend that Li *et al.* presents a "distance problem" amenable to solution by long-range PCR. However, as can be seen by objectively reviewing Li *et al. without* hindsight, the "distance problem" overcome by Li *et al.* was the roughly 30-43 bases that separated the nucleotide polymorphisms and prevented the use of allele-specific PCR-amplification for haplotyping the MN blood system.

Applicants offered evidence in the form of a Declaration Drs. McDonald and Evans on this point, as follows.

> Li *et al.* concludes with the following statement: "Although these alleles can be typed by allele-specific nested PCR following GPA-specific PCR, ASIP [allele specific inverse PCR], rather then nested PCR, can be applied to haplotyping of polymorphisms separated by a distance that is too long to be amplified by PCR." Li *et al.*, page 361, column 1. When the statement of Li *et al.* is placed in context, it is clear that Li *et al.* refers to haplotyping polymorphisms that are too distantly separated for the design of a single forward PCR reaction. To place the comment in context, one must consider the following two points: (1) The only problematic distance discussed by Li *et al.* is a separation of 30-40 base pairs which causes a primer design problem for forward allele-specific PCR, necessitating the use of allele-specific nested PCR. Li *et al.*, page 358, column 2. (2) Li *et al.* solves the problem by use of a circularization method that does not bring the polymorphisms closer (see paragraph 8, above) followed by inverse PCR. Conversely, Li *et al.* circularizes for the specific purpose of making the polymorphisms further apart so that AISP can be used rather than nested PCR. This is because the polymorphisms are too far apart for a single allele-specific primer to be designed for amplification, and too close together (30-43 base pairs) for a single nested PCR amplifications to produce a band that could be visualized on standard agarose/ethidium bromide gels (it is common knowledge that it is very difficult to visualize bands less than 100 base pairs on these gels). By making the polymorphisms further apart, a single ASIP reaction can be performed that produces products that are long enough (351 base pairs and 366 base pairs) to be visualized on agarose gels. This is demonstrated by the fact that Li *et al.* circularizes to make the polymorphisms further apart in one direction, followed by inverse PCR that amplifies products of 351 base pairs and 366 base pairs which are much longer than the 30-43 base pairs that separates C/T and G/T. If Li *et al.* had intended to bring polymorphisms closer together, they would have done so by circularizing to bring the polymorphisms closer together for a single PCR primer to be designed, and they would have then used a forward, not an inverse PCR, off of the circularized template. In contrast, Applicants' method is designed to haplotype polymorphisms that are distantly

located in *genomic* DNA by bringing the polymorphisms into closer proximity via circularization.

See **Appendix D**, Declaration of Drs. McDonald and Evans, filed March 22, 2004, ¶ 9.

In greater detail, Li *et al.* faced several problems when haplotyping the MN blood group system (the subject of their article). First, the polymorphisms Li *et al.* utilized to type the M^G, M^T, and N alleles of the GPA gene also occurred in the GPB and GPE genes, which are over 95% homologous with the GPA gene. Li *et al.* solved this by carrying out GPA-specific PCR. Li *et al.*, page 358, column 2. Second, the M^G, M^T and N alleles cannot be typed by the single allele-specific PCR amplification technique. Li *et al.*, page 358, column 1. Li *et al.* explains the problem as follows.

> Although primers specific to M^G and N alleles can be designed, the M^T-(and N-) specific nucleotide T in intron 1 is located *too far* (30-40 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T-specific primer.

Li *et al.*, page 358, columns 1-2 (emphasis added). The inability to design a single M^T-specific primer can be overcome by allele-specific nested PCR after GPA-specific PCR. Li *et al.*, page 361, column 1. However, Li *et al.* chose to use circularization and one round of ASIP, which allows for a *single* procedure using allele-specific primers. Li *et al.*, sentence spanning pages 360-1. Li *et al.* correctly notes on page 361 that multiple allele-specific PCRs (nested necessarily refers to multiple) can be performed for haplotype analysis after GPA-specific amplification, so the statement on page 361 refers back to the problem on page 358, in that "AISP [a single procedure]...can be applied to haplotyping polymorphisms separated by a distance that is too far to be amplified by PCR." It is my scientific opinion that this sentence refers to the fact that the polymorphisms are separated by a distance too large for a *single* primer to be designed that could anneal to a region that contains multiple polymorphisms (in intron1 and exon2 here) for a *single* allele-specific PCR procedure (and thus requiring either nested/multiple PCRs or Li *et al.*'s single AISP method). PCR here means a single PCR reaction, rather than if they had said nested PCR, which requires multiple PCR reactions. Thus, the problem of distance Li *et al.* refers to is polymorphisms being too far apart to design a primer for a single PCR procedure, and not a problem of polymorphisms being too far apart in genomic DNA for conventional haplotype analysis, as Li *et al.* notes that these polymorphisms can be haplotyped by RFLP and SSCP. Li *et al.* recognizes the following three problems of distance:

1. Too far for primer design for a single PCR reaction
2. Too close for visualizing short products of allele-specific PCRs on a gel

3. Having to perform multiple nested PCRs to overcome these 2 distance problems

> Li *et al.* circumvents the need for multiple nested PCRs by circularizing to make the polymorphisms farther apart, followed by a single ASIP procedure that does not require a primer to anneal to a region containing multiple polymorphisms and produces products that are long enough to clearly see on a gel.

See **Appendix D**, Declaration of Drs. McDonald and Evans, filed March 22, 2004, ¶ 10.

The Examiner dismisses the Declaration as an attempt "to place the quote in a context which is not correct because it ignores and distorts the plain meaning of Li..." and that "Li would not have said that the 30-40 bases between the polymorphisms was a distance too long to be amplified by PCR when he amplified a 357 base pair fragment." Office Action mailed 5/5/2004, page 10. However, as those of skill in the art know, there are multiple reasons why a PCR-based technique may not work, including primer design problems. Furthermore, Li *et al.* does discuss primer design problems caused by the 30-40 base pair distance between the polymorphisms as the reason that a single allele-specific PCR amplification (ASPA) technique cannot haplotype M^G, M^T, and N alleles. Specifically, on page 358, Li *et al.* state that "M^G, M^T, and N alleles can be typed by RFLP of SSCP analysis but not by the single ASPA technique." (RFLP and SSCP are both non-PCR techniques for haplotype analysis.) Li *et al.* explain that a 30-43 base pair distance causes this problem as follows.

> Although primers specific to M^G and N alleles can be designed, the M^T- (and N-) specific nucleotide T in intron 1 is located *too far* (30-40 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to **design a single M^T-specific primer**. Therefore, RFLP or SSCP analysis was performed to differentiate M^T from M^G and N alleles.

Li *et al.*, page 358, columns 1-2 (emphasis added).

In spite of the express statements of Li *et al*, the Examiner maintains that "Li never discusses the need to design a single primer for multiple polymorphisms and this is not what allele specific PCR is all about." Office Action dated 5/5/2004, page 10. However, as shown in the quote, above, Li *et al. do* discuss the need to design "a single M^T-specific primer;" the M^T allele is composed of multiple polymorphisms. Accordingly, Li *et al. does* discuss a single allele specific primer for multiple polymorphisms. As Li *et al.* explain, their ASIP technique obviates

the need for such a primer and allows the use of two allele-specific primers. The objective evidence of record supports a conclusion that "distance problem" overcome by Li *et al.* was the roughly 30-43 bases that separated the nucleotide polymorphisms and prevented the use of allele-specific PCR-amplification for haplotyping the MN blood system. No other distance problem is mentioned anywhere in Li *et al.*

In the Office Action mailed 5/5/2004, the Examiner reasoned that "[Li *et al.*] expressly teaches separation that is too far apart for standard PCR" and concludes that "[s]ince Standard PCR can easily amplify 1000 or more bases, Li is discussing polymorphisms that are at least that distant...." See the Office Action, page 10. Applicants have previously pointed out that for this to be so, Li *et al.* must refer *for the first time* to polymorphisms at least 1,000 base pairs apart *in the final sentence of their paper.* Applicants also pointed out that nothing in Li *et al.* supports the Examiner's conclusion and that it must be based solely on the Examiner's knowledge. Accordingly, Applicants invited the Examiner to provide an affidavit under 37 CFR 1.104(d)(2). See the response mailed March 22, 2004, page 6. This was not done. Instead, the Examiner replied that "Applicant then argues that standard PCR is not commonly understood as being capable of amplifying 1000 bases" and that "no declaration from the examiner is needed to prove this point." Office Action mailed 5/5/2004, page 12. Applicants never argued that standard PCR is incapable of amplifying 1,000 or more bases. Rather, Applicants' filed an affidavit stating that in their scientific opinion, the final sentence of Li *et al.* refers to the distance problem that was actually discussed earlier in the reference (30-43 base pairs preventing the design of a single allele specific PCR primer). Because the Examiner has not filed an affidavit, there remains no evidence of record to support the Examiner's conclusion that Li *et al.* suggested applying their technique to haplotype polymorphisms at least 1,000 base pairs apart.

As stated above, the objective evidence supports a conclusion that Li *et al.* were discussing a situation in which a single allele specific PCR primer cannot be designed. It is through the application of hindsight, informed by Applicants' own disclosure, that the Examiner reads Li *et al.* to refer haplotype structures widely separated by hundreds or thousands of base pairs. A single line in a prior art reference should not be taken out of context and relied upon with the benefit of hindsight to show obviousness. *See, e.g., Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 796 F.2d 443, 230 U.S.P.Q. 416 (Fed. Cir. 1986). However, this is

exactly what the Examiner has done by injecting his own hindsight-driven interpretation into the final sentence of Li *et al.*

When Li *et al.* is read objectively in light of its own teachings without consideration of Applicants' disclosure, it is clear that the last sentence refers to the actual distance problems (30-43 base pairs) overcome using ASIP. For this reason, Li *et al.* does not provide the motivation for its combination with, or modification by, the long-range PCR techniques used by Michalatos-Beloin *et al.*

c. The Examiner's Responses Fail to Provide a Basis for Combining Patel et al. With the Other References

The Examiner maintains that "Patel teaches that inverse PCR methods such as those used by Li can be applied to haplotype sequences up to 10 kb apart...." Office Action mailed 5/5/2004, page 5. The page of Patel *et al.* cited by the Examiner does generally state that "others have successfully applied inverse PCR to genomic regions of over 10 kb," a statement that provides nothing more than the motivation to try inverse PCR with genomic regions of over 10 kb as the final step of the method of Patel *et al.* There is no motivation for the combination of Patel *et al.* with dissimilar methods such as Li *et al.* or Michalatos-Beloin *et al.*

As discussed above, Patel *et al.* involves the digestion of total genomic DNA with a first restriction enzyme, circularization of the DNA, re-linearization of the circular molecules using a second restriction enzyme, ultimately followed by an inverse PCR technique upon the re-linearized genomic DNA molecules. Patel *et al.* never discusses the method found in Li *et al.*, which involves generating a linear heterozygous PCR product from a genomic DNA segment, circularizing the segment by intramolecular ligation, and then performing ASIP on the circularized molecule. Thus, there is no motivation in Patel *et al.* to combine these two dissimilar methods.

On page 14 of the Office Action mailed 5/5/2004, the Examiner asserts that dissimilarities between Patel *et al.* and the other cited references are "not a patentable distinction." This statement serves only to add confusion to the rejection. The issue is not whether Patel *et al.* is patentably distinct from the other references. The Examiner has rejected Applicants' claimed method over Li *et al.* in combination with Patel *et al* and Michalatos-Beloin

et al. The relevant issue is whether Patel *et al.* provides motivation for the combination of references asserted by the Examiner. It does not, in part because of the dissimilarities between it and the other references.

The Examiner also states on page 14 of the Office Action that "Patel is present solely to show that genomic DNA can be a source of DNA for the ASIP method of Li" but this is irrelevant to the rejected claims, which do not recite inverse PCR from genomic DNA.

Further, to the extent that Patel *et al.* is cited with respect to the ASIP step of Li *et al.*, Applicants note that the ASIP step is the final, allele-specific inverse PCR step that Li *et al.* use for haplotyping. This step is irrelevant to the patentability of Applicants' claimed method, which does not require allele-specific inverse PCR for determining haplotype. See independent claim 1, which does not recite any specific haplotype determining technique. Whether or not genomic DNA could be a substrate for the allele-specific inverse PCR step of Li *et al.* is an issue wholly irrelevant to the patentability of Applicants' invention.

d. The Examiner's Responses Overlook Fundamental Differences Between Michalatos-Beloin et al. and the Other References

The Examiner maintains that Michalatos-Beloin *et al.* "teaches haplotyping where the molecules are prepared by long range PCR" and that an "ordinary practitioner would have been motivated to use long range PCR to prepare the template for the method of Li...." Office Action mailed 5/5/2004, page 6. To the contrary, the technique of Michalatos-Beloin *et al.* is a one-step PCR technique where haplotyping is accomplished in the same PCR step by which hemizygous target DNA is isolated. Thus, the hemizygous target DNA isolated by Michalatos-Beloin *et al.* would not be suitable for the technique of Li *et al.* which relies upon the isolation of a *heterozygous* PCR product.

The Office Action overlooks this fundamental difference, relying upon the Michalatos-Beloin *et al.* statement that "The allele-specific long range PCR products were used as templates for amplification of the STR." As explained on page 4867 of Michalatos-Beloin *et al.*, the amplification of a short terminal repeat (STR) from the target DNA was a control PCR used to confirm that the one-step long-range PCR technique correctly isolated hemizygous target DNA. As discussed above in section 1,d, Michalatos-Beloin *et al.* touts the advantage of accomplishing

the isolation of hemizygous DNA *and* haplotyping in one step via its PCR protocol (on page 4867, column 2). It thereby teaches away from its combination with methods that require additional steps such as Li *et al.* (or Patel *et al.*, for that matter). The Examiner's responses have not identified any motivation for the modification of Michalatos-Beloin *et al.* to accommodate the additional, different steps of Li *et al.*

3. The Examiner Seeks to Impose a Requirement that the References be Combined Based Upon Only Subjective Assertions

In his response, the Examiner states that Applicants are conducting a "piecemeal examination of the references..." and asserts that the references must be taken as a whole. Office Action mailed 5/5/2004. However, the burden of establishing a *prima facie* case of obviousness falls upon the Examiner who "can satisfy this burden only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references." *In re Fritch*, 972 F.2d 1260, 1265, 23 U.S.P.Q.2d 1780, 1783 (Fed. Cir. 1992). "Combining prior art references without evidence of such a suggestion, teaching, or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability—the essence of hindsight." *In re Dembicazak*, 175 F.3d 994, 999, 50 U.S.P.Q.2d 1614, 1617 (Fed. Cir. 1999). For the reasons stated above, the Examiner relies upon subjective and conclusory arguments about the teachings of the references for motivation to combine and modify. Until the Examiner demonstrates an objective basis for the combination of Li *et al.* with Patel *et al* and Michalatos-Beloin *et al.* the references are not properly considered as a whole.

4. The Examiner Has Improperly Dismissed Applicants' Declaration Without Submitting Counter Evidence.

Applicants have drawn to the Examiner's attention to the points at which his conclusions are contrary to the objective evidence of record, including the following:

- Li *et al.* teaches a method that does not bring the relevant polymorphisms into closer proximity. See Li *et al.*, Figure 1.

- The only distance problem discussed in Li *et al.* relates to a 30-43 base pair separation between polymorphisms that prevents the design of a single allele specific primer for the M^T allele. Li *et al.*, page 358, col. 2, line 11.

- Li *et al* actually solves the only distance problem they discuss (by use of ASIP). Li *et al.*, page 358, col. 2, lines 18-22 and page 360, col. 1, lines 5-11.

- The final sentence of Li *et al.* is logically read to refer to the problem caused by the 30-43 base pair separation between polymorphisms. Li *et al.*, page 358, paragraphs spanning cols. 1-2 and page 361, col. 1.

Applicants' have filed a Rule 132 Declaration to this effect. See **Appendix D**, Declaration of Drs. McDonald and Evans, filed March 22, 2004, ¶ 10.

Rather than providing countervailing evidence in reply to Applicants' Declaration, the Examiner has attempted to brush aside this evidence with argument (previously discussed herein, sections 2a & 2b.) Despite the invitation to do so, the Examiner has not bolstered his rejection with evidence in the form of additional references or an affidavit under 37 CFR 1.104(d)(2) to support his conclusions. See, e.g., Applicants' response filed March 22, 2004. This is improper because rejection must be supported by evidence, not conclusory assertions about the general knowledge of those of skill in the art. *See In re Lee* at page 1435. The evidence of record does not support the combination of references and the rejection should be reversed.

5. *Even if Motivation to Combine Were Established, the References Cited by the Examiner Do Not Teach Applicants' Invention.*

As noted above, Li *et al.* teaches a method that does not bring the nucleotide polymorphisms into closer proximity on the circular molecule. Patel *et al.* teaches a method in which the target template for haplotyping is not even prepared by PCR. Michalatos-Beloin *et al.* teaches a one-step haplotyping PCR that produces hemizygous DNA. Applicants emphasize that, even when considered together (and there is no motivation to do so), the combination of Li *et al.*, Patel *et al.*, and Michalatos-Beloin *et al.* does not teach or suggest a method for determining the haplotype structure of a contiguous DNA segment having at least two nucleotide polymorphisms separated by at least 200 nucleotides comprising the steps of (1) obtaining a DNA sample comprising the contiguous DNA segment; (2) using the DNA sample as a template for polymerase chain reaction (PCR) amplification of a DNA fragment comprising the

DNA segment; (3) ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule, wherein the first nucleotide polymorphism and the second nucleotide polymorphism are brought into closer proximity on the circular DNA molecule relative to the contiguous DNA segment; and (4) determining the haplotype of the first nucleotide polymorphism and the second nucleotide polymorphism. It is Applicants' disclosure that teaches this a method. As discussed above, the record is devoid of objective evidence for the modification of the references the Examiner implies one of skill in the art would undertake.

For all of these reasons, the rejection of claims 1-18 and 21-22 should be withdrawn.

B. The Rejection of Claim 12 Cannot be Upheld.

Besides the deficiencies enumerated above, the rejection of claim 12 is further defective: Claim 12 is also drawn to the method of claim 1 wherein the haplotype of the first nucleotide polymorphism and the second nucleotide polymorphism on the circular DNA molecule is detected by restriction fragment analysis of the circularized segment or of a PCR amplification product using the circular DNA molecule as a template. The Examiner has failed to cite any reference that shows utilizing such a technique after ligation and circularization. Rather, it is Applicants' own disclosure that provides this teaching. Because of this additional defect, the rejection of claim 12 should be withdrawn.

C. The Rejection of Claim 17 Cannot be Upheld.

Claim 17 is rejected under 35 U.S.C. § 103 over Li *et al.* in combination with Patel *et al.* and Michalatos-Beloin *et al.* in further view of Krynetski *et al.* (1995) *Proc. Natl. Acad. Sci.*, 92:949-953. As described above, there is no motivation to combine Li *et al.* with Patel *et al.* and Michalatos-Beloin *et al.* Krynetski *et al.* merely teaches a point mutation of the TPMT gene and does not satisfy the deficiencies of the primary references. Consequently, the motivation to combine these four references has not been established and the rejection should be withdrawn.

D. The Rejection of Claim 18 Cannot be Upheld.

Claim 18 is rejected under 35 U.S.C. § 103 over Li *et al.* in combination with Patel *et al.* and Michalatos-Beloin *et al.* in further view of Martin *et al.* (2000) *Am. J. Hum. Genet.*, 67:383-394. There is no motivation to combine Li *et al.* with Patel *et al.* and Michalatos-Beloin *et al.* Martin *et al.* teaches SNPs in the region surrounding the APOE gene but does not satisfy the deficiencies of the primary references. Accordingly, the motivation to combine these four references has not been established and the rejection should be withdrawn.

E. The Rejection of Claim 22 Cannot be Upheld.

Claim 22 specifies that DNA sequence immediately 5' to the first nucleotide polymorphism that encompasses an annealing site for a primer and DNA sequence immediately 3' to the second nucleotide polymorphism that encompasses an annealing site for a primer and that the length of these sequences is selected from less than 500, less than 400, less than 300, less than 200, less than 100, or less than 50 bases. None of the references teach or suggest such a selection step. Because the references do not teach or suggest this step, the rejection of claim 22 should be withdrawn.

Conclusion

In the present case, the Examiner has failed to establish by objective evidence the required motivation to combine the references cited in the rejection. In particular, the Examiner has failed to show that the art worker would be motivated to apply Li *et al.* to determining the haplotype structure of a DNA segment comprising widely separated nucleotide polymorphisms because Li *et al.* does not teach or suggest bringing the polymorphisms into closer proximity. Further, the art worker would not be motivated to modify Li *et al.* in a way that would bring the polymorphisms into closer proximity: the technique was not designed to solve such a problem. With respect to the Examiner's contention that Li *et al.* provide the motivation to apply its technique to widely separated polymorphisms in the form of an unsolved problem, this is not the proper standard. The record must show motivation to combine the references in a way that would produce the invention claimed, not the motivation to solve a problem. The secondary references fail to cure the deficiencies of the primary reference.

Applicants explained that the rejection relies upon Applicants' own teaching to provide the motivation for both the modification and combination of references asserted. The Examiner has responded that Applicants are improperly arguing against the references individually instead of as a whole. However, the references should not be taken as a whole because the Examiner has not established the motivation for their combination by objective evidence. Further, even if the references were combined, they do not teach or suggest Applicants' invention. The Examiner has also improperly dismissed Applicants' Rule 132 Declaration without providing counter evidence. Thus, none of the Examiner's responses cure the deficiencies of the Section 103 rejection of claims 1-18 and 21-22. The rejection should be withdrawn.

In addition to the deficiencies listed above, the Examiner fails to cite any reference for the teaching or suggestion to use Applicants' method with the additional step of RFLP analysis as recited in claim 12. The rejection of claim 12 should be withdrawn for this deficiency, as well as those listed above.

The Examiner fails to provide evidence of motivation to combine the Li *et al.*, Patel *et al.*, and Michalatos-Beloin *et al.* with the additional reference cited against claim 17. The rejection of claim 17 should be withdrawn due to this deficiency, as well as the deficiencies

listed above. Similarly, the Examiner cites an additional reference against claim 18 without identifying objective evidence for the combination and the rejection should be withdrawn, accordingly.

Finally, no reference is cited for teaching or suggesting the selection step of claim 22, and the rejection must be withdrawn on this basis, as well those set forth above.

For all of these reasons, the rejections should be reversed.

Respectfully submitted,

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Nora C. Martinez

CLAIMS

1. (Previously Presented) A method for determining the haplotype structure of a contiguous DNA segment comprising a first nucleotide polymorphism (NP) and a second NP separated by at least 200 nucleotides, said method comprising:

(a) obtaining a DNA sample comprising said contiguous DNA segment;

(b) using said DNA sample as a template for polymerase chain reaction (PCR) amplification of a DNA fragment comprising said contiguous DNA segment,

wherein the PCR amplification is performed with

a first primer capable of annealing to a region adjacent to the first NP and distal to the second NP and

a second primer capable of annealing to a region adjacent to the second NP and distal to the first NP;

(c) ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule, wherein said first NP and said second NP are brought into closer proximity on said circular DNA molecule relative to said contiguous DNA segment; and

(d) determining the haplotype of said first NP and said second NP.

2. (Original) The method of claim 1 wherein said first NP and said second NP are separated by at least 1000 nucleotides.

3. (Original) The method of claim 2 wherein said first NP and said second NP are separated by at least 10,000 nucleotides.

4. (Original) The method of claim 3 wherein said first NP and said second NP are separated by at least 30,000 nucleotides.

5. (Original) The method of claim 1 wherein said first NP and said second NP are selected from the group consisting of a substitution of five nucleotides or less, a deletion of five nucleotides or less, and an insertion of five nucleotides or less.

6. (Original) The method of claim 5 wherein said first NP and said second NP each consist of a single nucleotide substitution.

7. (Original) The method of claim 1 wherein one or more additional NPs are located between said first NP and said second NP.

8. (Original) The method of claim 7 comprising the additional step of determining the haplotype of said one or more additional NPs.

9. (Original) The method of claim 1 wherein said nucleic acid sample is from a human source.

10. (Original) The method of claim 1 wherein the fragment of step (b) is obtained by amplification of said segment from said DNA sample using long-range polymerase chain reaction (LR-PCR).

11. (Original) The method of claim 1 wherein the fragment of step (b) is cleaved using a restriction enzyme that does not cleave any nucleotide sequences occurring between said first NP and said second NP on said contiguous DNA segment.

12. (Original) The method of claim 1 wherein the haplotype of said first NP and said second NP on said circular DNA molecule is detected by restriction fragment analysis of said circularized segment or of a PCR amplification product using said circular DNA molecule as a template.

13. (Original) The method of claim 1 wherein the haplotype of said first NP and said second NP is detected by PCR amplification using primers whose ability to amplify segments from said circular DNA molecule is dependent upon the presence or absence of a particular haplotype at said first NP and said second NP.

14. (Original) The method of claim 1 wherein said first NP and said second NP are located in the same gene.

15. (Original) The method of claim 14 wherein the haplotype of each allele of said gene is determined.

16. (Original) The method of claim 14, wherein at least one of said first NP and said second NP is associated with a clinically relevant phenotype.

17. (Original) The method of claim 14, wherein said gene is the TPMT gene.

18. (Original) The method of claim 14, wherein said gene is selected from the group consisting of genes encoding beta2 receptor, apoE, OPRM1, and IL-4 receptor alpha.

19-20 (Canceled)

21. (Previously Presented) A method for determining the haplotype structure of a contiguous DNA segment comprising a first nucleotide polymorphism (NP) and a second NP separated by at least 200 nucleotides, said method comprising:

(a) obtaining a DNA sample comprising said contiguous DNA segment, wherein the DNA segment further comprises

a DNA sequence immediately 5' to the first NP that encompasses an annealing site for a primer and

a DNA sequence immediately 3' to the second NP that encompasses an annealing site for a primer;

(b) using said DNA sample as a template for polymerase chain reaction (PCR) amplification utilizing said primers of a DNA fragment comprising said contiguous DNA segment;

(c) ligating the ends of said DNA fragment to each other so as to produce a circular DNA molecule, wherein said first NP and said second NP are brought into closer proximity on said circular DNA molecule relative to said contiguous DNA segment; and

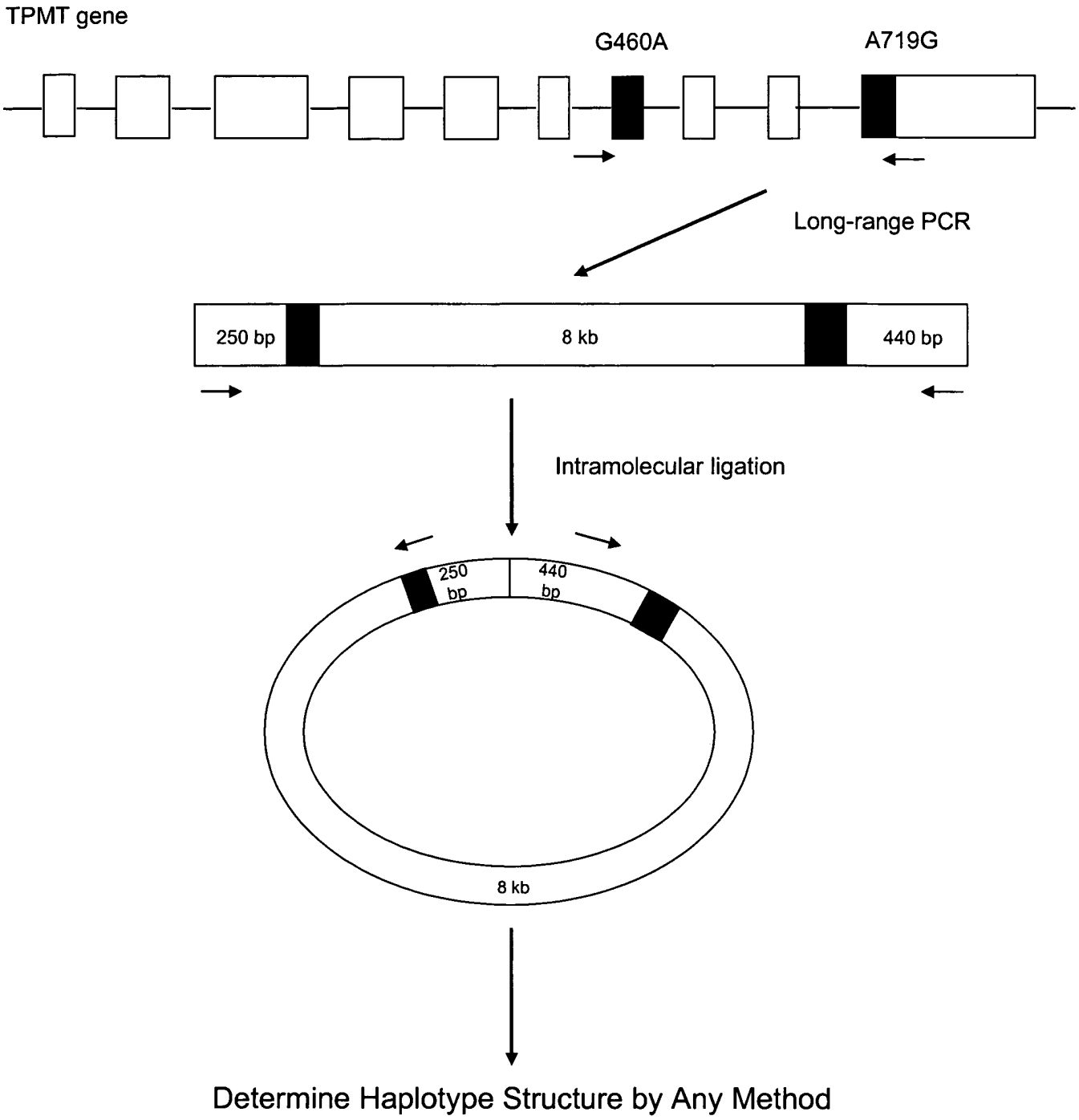
(d) determining the haplotype of said first NP and said second NP.

22. (Previously presented) The method of 21, wherein the DNA sequence immediately 5' to the first NP has a length selected from the group consisting of:

less than 500, less than 400, less than 300, less than 200, less than 100, or less than 50 bases long; and,

wherein the DNA sequence immediately 3' to the second NP has a length selected from the group consisting of:

less than 500, less than 400, less than 300, less than 200, less than 100, or less than 50 bases long.



Allele-Specific, Inverse-PCR Amplification for Genotyping MN Blood Group

BioTechniques 25:358-362 (September 1998)

The MN blood group system, usually typed serologically, is based on polymorphic red cell antigens. Glycophorin A (GPA), a sialoglycoprotein on the erythrocyte membrane, carries M and N antigens, based on two changes of the amino acid sequence at positions 1 and 5 (M, serine and glycine; N, leucine and glutamic acid). M and N alleles of the *GPA* gene are attributed to three nucleotide substitutions in exon 2 (Figure 1) (10,18) and are determined by restriction fragment-length polymorphism (RFLP) analysis (1,6,12), single-strand conformation polymorphism (SSCP) analysis of the polymerase chain reaction (PCR) products (1) or allele-specific PCR amplification (ASPA) (4,8,13). MN genotyping by DNA analysis is informative for forensic identification and in several clinical situations where serological phenotyping is difficult or impossible (8), such as *(i)* in a patient transfused with large amounts of blood from various donors, *(ii)* a fetus at risk for hemolytic disease and *(iii)* a patient with autoimmune hemolytic anemia. A G/T substitution in intron 1 of the *GPA* gene between M and N alleles (10) was found among conventional (serological) M alleles, being divided into M^G and M^T alleles (Figure 1) (1). All N alleles have the nucleotide T in that position. This G/T substitution may not affect the antigenicity. The frequencies of M^G and M^T alleles in the Japanese population were 0.547 and 0.040. The heterozygosity, the polymorphism information content and the probability of paternity exclusion of this improved MN system were 0.529, 0.425 and 0.232, respectively; whereas, those of the conventional MN system were 0.485, 0.367 and 0.184, respectively (1). Therefore, subtyping of M alleles may be informative for forensic and clinical purposes. M^G, M^T and N alleles can be typed by RFLP or SSCP analysis but not by the single ASPA technique. Because M^T and N-specific sequences are also observed in glycophorin E (*GPE*) and glycophorin B (*GPB*) genes, respectively (Figure 1), which are more than 95% homologous in exons and introns with the *GPA* gene, one primer for ASPA should be GPA-specific to avoid coamplification of GPB and GPE segments. Although primers specific to M^G and N alleles can be designed, the M^T- (and N-) specific nucleotide T in intron 1 is located too far (30–43 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T-specific primer. Therefore, RFLP or SSCP analysis was performed to differentiate M^T from M^G and N alleles (1). Here, a novel method for genotyping the MN blood group was performed using allele-specific, inverse-PCR (ASIP) following GPA-specific PCR amplification.

GPA-Specific PCR

The GPA fragment (357 bp) was amplified using two GPA-specific primers: MN-FF (5′-GAG GGA ATT TGT CTT TTG CA-3′) and MN-CR (5′-AGA GGC AAG AAT TCC TCC-3′) (Reference 10; Figure 1a). The PCR mixture (25 µL) consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.01 U/µL of AmpliTaq Gold™ DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 15 ng of template DNA. PCR consisted of preheating at 95°C for 9 min; 40 cycles of 95°C for 40 s, 60°C for 40 s and 72°C for 40 s; followed by a post-incubation step at 72°C for 2 min.

Ligation of the Amplified Fragment

The PCR products (0.5 µL each) were added to 100 µL of ligation mixture consisting of 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 66 µM ATP and 0.02 U/µL of T4 DNA Ligase (Toyobo, Osaka, Japan), followed by incubation at 16°C overnight.

Allele-Specific PCR

The ligation mixture (0.5 µL) was added into the PCR mixture (25 µL) containing 0.01 U/µL of *Taq* DNA Polymerase (Boehringer Mannheim, Mannheim, Germany) and one of allele-specific primer sets: MN-MF (5′-GCA TCA AGT ACC ACT GGT-3′) and MN-GR (5′-GCT CAC AAT TGC TGT ATA AC-3′) for amplification of 351-bp M^G, or MN-MF, MN-NF (5′-att gtg agc ATA TCA GCA TT-3′) and MN-TR (5′-gct cac aat TGC TGT ATA AA-3′) for 351-bp M^T and 366-bp N sequences, respectively (Figure 1b). The annealing sites of MN-NF and MN-TR complementarily overlapped for 9 bp (shown by small letters in primer sequences). PCR consisted of preheating at 95°C for 2 min; 30 cycles of 94°C for 40 s, 63°C for 40 s and 72°C for 40 s; followed by a post-incubation step at 72°C for 2 min. The PCR products were electrophoresed on a 6% native polyacrylamide gel, followed by staining with ethidium bromide.

Genotypes M^GM^G, M^GM^T, M^GN, M^TN and NN typed by ASIP (Figure 2) completely corresponded to those typed by the PCR-RFLP method (1). This result suggested that intermolecular ligation producing wrong alleles (for example, M^T-specific, 351-bp fragment amplified following intermolecular ligation between 3′ end of M^G and 5′ end of N fragments) did not occur, but that intramolecular ligation of each allele occurred under the experimental conditions described above. The annealing sites of the primers MN-NF and MN-TR overlapped for 9 bp, which meant that the annealing site for the reverse primer in the PCR product from the 357-bp circular template annealed with the forward primer was 9 bp shorter than the reverse primer. However, fragments of the expected length (366 bp) were amplified from N alleles. This was likely because the first PCR products contained little nontarget sequences. Consequently, the reverse primer annealed to the 9-bp shorter annealing site of the 357-bp target sequence, resulting in the duplication of the 366-bp fragment. Therefore, the fragment acted as a complete template.

The second inverse-PCR using a short circular template was performed with the *Taq* DNA polymerase (Boehringer Mannheim), which is a mutant *Taq* polymerase lacking 5′ to 3′ exonuclease activity (16) [as does the Stoffel fragment (11)] to avoid cleaving

the annealed primer and its extension after duplication of the whole sequence. The polymerase lacking 5′ to 3′ exonuclease activity was suggested to permit efficient amplification of long fragments (7).

For ASIP analysis, intramolecular ligation is absolutely required. If intermolecular ligation between different alleles occurred dominantly, the wrong alleles or haplotypes would be detected, and polymorphism analysis would end in failure. Linear or circular products of intermolecular or intramolecular ligation, respectively, are predicted according to the DNA concentration (5); intramolecular ligation will occur dominantly when the $[51.1/(\text{concentration})]^2/(\text{molecular weight})$ value is >2. For intramolecular ligation of a 357-bp fragment, the ligation mixture should include <6.8 μg per 100 μL of the fragment. PCR usually generates a product <1 μg per 5 μL reaction volume, because the amount of product reaches a plateau during multiple cycles of amplification (9). Therefore, intramolecular ligation was expected by adding 0.5 μL of the first PCR product to 100 μL of the ligation mixture, and the results shown in Figure 2 suggest that predominantly intramolecular ligation occurred under these experimental conditions.

However, when one-tenth of the amount (equivalent to 0.05 μL) of the first PCR product was added to 100 μL of the ligation mixture, nonspecific fragments were detected by ASIP, which interfered with the genotyping (data not shown). *Taq* DNA polymerase is known to catalyze non-templated addition of a nucleotide (principally adenosine) to 15%–90% of the 3′ ends of PCR products, according to the sequence on the 5′ end of reverse primers (2,3,15). Adenylation of blunt ends of PCR products resulting in noncomplementary 3′-stalk termini inhibits intramolecular ligation. This indicates that only a portion of the first PCR products acted as templates for the second PCR, and the addition of 0.05 μL of the first PCR products into the ligation mixture likely resulted in a lack of templates for the second PCR, inducing nonspecific amplification. In this study, the first PCR was performed using AmpliTaq Gold, so that the amount of product would reach a plateau. Because this enzyme is activated by heat, nonspecific amplification occurring at low temperatures can be suppressed, and more cycles can be added to the typical PCR (consisting of 25–30 cycles) to increase the yield of specific products (according to the manufacturer's instruction). The efficiency of ligation may be increased following restriction digestion of both ends of the first PCR products to generate complementary termini. For this purpose, the restriction sites can be inserted into the 5′ end of primers for the first PCR. However, this ASIP method did not include the restriction step to simplify the procedure.

Inverse PCR, or inside-out PCR, was explored to analyze unknown sequences that flank a region of known sequence (14,17). This technique, which involves ligation of separated regions at the ends of a sequence (a restriction fragment or a PCR product), can also be applied to closely linked polymorphisms. Haplotypes consisting of heterozygous polymorphisms have been determined by pedigree analysis. However, using inverse PCR, the linked

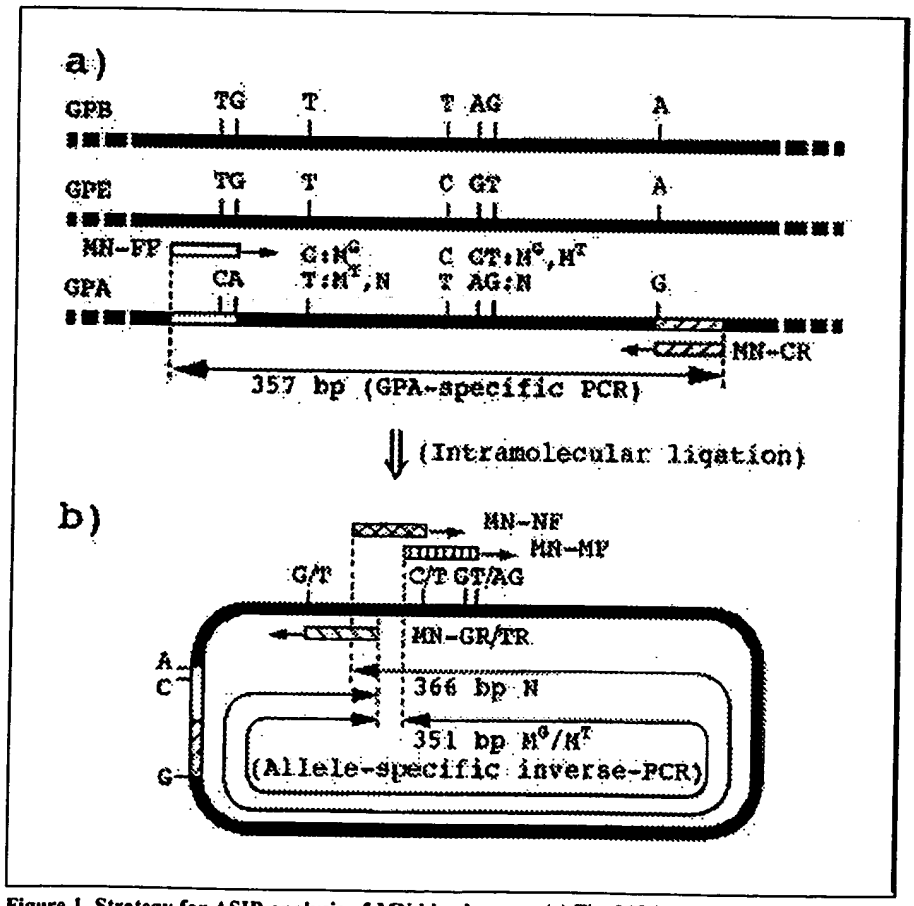


Figure 1. Strategy for ASIP analysis of MN blood group. (a) The 357-bp sequences including nucleotide substitutions specific to M^G, M^T and N alleles are amplified using GPA gene-specific primers MN-FF and MN-CR. (b) Following intramolecular ligation of the amplified fragments, PCR amplification is carried out using an allele-specific primer set, MN-MF and MN-GR for M^G, or MN-MF, MN-NF and MN-TR for M^T and N alleles. The annealing sites of 5′ ends of MN-NF and MN-TR overlapped complementarily for 9 bp, being predicted to produce 366-bp N fragment from 357-bp circular template.

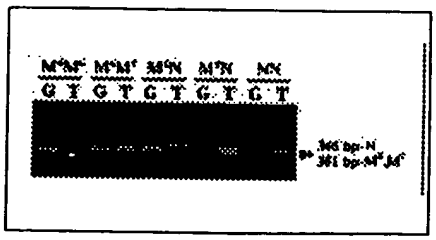


Figure 2. ASIP analysis of MN genotypes M^GM^G, M^GM^T, M^GN, M^TN and NN. Lanes marked G show amplification of 351-bp M^G allele using primers MN-MF and MN-GR. T lanes show amplification of 351-bp M^T and/or 366-bp N allele(s) using primers MN-MF, MN-NF and MN-TR.

polymorphisms can be analyzed by a single procedure using allele-specific primers. In this study, M^G, M^T and N alleles were regarded as haplotype alleles and analyzed by ASIP. Although these alleles can be typed by allele-specific nested PCR following GPA-specific PCR, ASIP, rather than nested PCR, can be applied to haplotyping of polymorphisms separated by a distance that is too long to be amplified by PCR.

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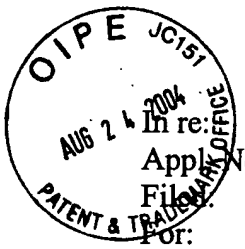
Attorney's Docket No. 44158/209598(5853-3) PATENTS



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Evans et al.	Confirmation No.:	2302
Appl. No.:	09/829,113	Group Art Unit:	1634
Filed:	April 9, 2001	Examiner:	Jeffrey N. Fredman
For:	HAPLOTYPING METHOD FOR MULTIPLE DISTAL NUCLEOTIDE POLYMORPHISMS		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COPY

RULE 37 C.F.R. § 1.132 DECLARATION
of Oliver Gene McDonald and William Evans

I, Oliver Gene McDonald and I, William Edward Evans, do hereby declare and say as follows:

1. I, Oliver Gene McDonald, am an inventor of the subject matter of the above-captioned application.

2. I, Oliver Gene McDonald, am skilled in the art of the field of the invention. I am pursuing a M.D./Ph.D. in molecular physiology from University of Virginia. I have a Bachelor of Science degree in biology from University of Tennessee. I have doctoral training from Dr. Gary K. Owens of University of Virginia. Since 2001, I have been engaged in the study of molecular biology, and particularly of genomic DNA. I have been employed by St. Jude from 2000-2002, and was in their Pharmacogenetics Group.

3. I, William Edward Evans, am an inventor of the subject matter of the above-captioned application.

4. I, William Edward Evans, am skilled in the art of the field of the invention. I have a Pharm.D. from University of Tennessee, Memphis. I have a Bachelor of Science degree in Pharmacology from University of Tennessee. I have doctoral degree in Clinical

Pharmacology from the University of Tennessee. Since 1987, I have been engaged in the study of molecular biology, and particularly of Pharmacogenetics and Pharmacogenomics. I have been employed by St. Jude since 1976, and have been the Scientific Director since 2001.

5. I have read and understood the Office Actions in the above case dated 12 December 2002, 7 July 2003, and 24 November 2001. I have also read and understood references cited and discussed in this case, including Li *et al.* (1998) *BioTechniques* 25:358-361, Patel *et al.* (1991) *Nucleic Acids Res.*, 19:3561-3567, and Michalatos-Beloin *et al.* (1996) *Nucleic Acids Res.* 24:4841-4843.

6. The method of Patel *et al.* (1991) *Nucleic Acids Res.*, 19:3561-3567 is conceptually and technically different from our method. These differences include the fact that our claimed method isolates the target sequence from genomic DNA before circularization (ligation) by amplifying a DNA fragment by PCR. In contrast, the Patel *et al.* method isolates the target sequence after circularization of total genomic DNA. Further, our claimed method involves isolation of target sequence from undigested and unligated genomic DNA by PCR techniques, including long range PCR. In contrast, Patel *et al.* isolates target sequence via short-range Allele-specific PCR from digested and ligated genomic DNA and production of a hemizygous target sequence. See the final slide of our Communication filed February 11, 2003 (hereinafter "Slide 4," provided for the reader's convenience as Appendix A of the response filed concurrently herewith).

7. The method of Michalatos-Beloin *et al.* (1996) *Nucleic Acids Res.* 24:4841-4843 is conceptually and technically different from our method. These differences include the fact that our initial isolation of the target sequence produces heterozygous target sequence, whereas Michalatos-Beloin *et al.* produces hemizygous DNA. Thus, we are not limited to haplotype analysis via isolation of hemizygous DNA. Michalatos-Beloin *et al.* themselves emphasized that their method produces hemizygous DNA by stating at page 4867, column 2, that "the ability to isolate hemizygous DNA segments from heterozygous genomes via molecular haplotyping will provide the accuracy necessary in these diverse applications." See the third slide of our

Communication filed February 11, 2003 (hereinafter "Slide 3," provided for the reader's convenience as Appendix A of the response filed concurrently herewith).

8. The method of the newly cited reference to Li *et al.* is also conceptually different from our claimed method. In my scientific opinion, Li *et al.* is distinguishable from our method on grounds including the following points.

- Li *et al.* does not circularize in a way that brings polymorphisms closer together the way our claimed method does. Figure 1 of our application and Figure 1 of Li *et al.* clearly demonstrate this result. When in linear genomic DNA, the distal-most polymorphisms, M-G/T in intron 1 and M(G/T)-N(AG) in exon 2, are separated by 30-43 base pairs (page 358, second column, line 12). Once circularized by Li *et al.*, the polymorphisms are the *same* distance from each other in one direction of the circle and *farther apart* in the other direction (a 357 base pairs linear molecule was circularized, thus 357 minus 30-43 gives the polymorphisms a distance of between 314 to 327 base pairs from each other).

- Li *et al.* does not provide the advantages of our method. By using inverse PCR, Li *et al.* simply generate back the same, or nearly the same, linear molecule that was circularized by using inverse PCR (after inverse PCR off of a 357 base pairs circle, Li *et al.* generate a 351 and a 366 base pairs fragment). In my scientific opinion, had Li *et al.* intended haplotype analysis of circular molecules the way our method allows (i.e. aimed at analyzing polymorphisms that had been brought into closer proximity), then Li *et al.* would *not* have used inverse PCR because inverse PCR simply generates back the same, or nearly the same, linear molecule that was circularized in the first place.

Because of these differences, one would not combine Li *et al.*'s method with Patel *et al.* or Michalatos-Beloin *et al.* Further, because of these differences, the combination of these dissimilar methods would not result in a workable method.

9. Li *et al.* concludes with the following statement: "Although these alleles can be typed by allele-specific nested PCR following GPA-specific PCR, ASIP [allele specific inverse PCR], rather then nested PCR, can be applied to haplotyping of polymorphisms separated by a distance that is too long to be amplified by PCR." Li *et al.*, page 361, column 1. When the statement of Li *et al.* is placed in context, it is clear that Li *et al.* refers to haplotyping polymorphisms that are too distantly separated for the design of a single forward PCR reaction. To place the comment in context, one must consider the following two points: (1) The only problematic distance discussed by Li *et al.* is a separation of 30-40 base pairs which causes a primer design problem for forward allele-specific PCR, necessitating the use of allele-specific nested PCR. Li *et al.*, page 358, column 2. (2) Li *et al.* solves the problem by use of a circularization method that does not bring the polymorphisms closer (see paragraph 8, above) followed by inverse PCR. Conversely, Li *et al.* circularizes for the specific purpose of making the polymorphisms further apart so that AISP can be used rather than nested PCR. This is because the polymorphisms are too far apart for a single allele-specific primer to be designed for amplification, and too close together (30-43base pairs) for a single nested PCR amplifications to produce a band that could be visualized on standard agarose/ethidium bromide gels (it is common knowledge that it is very difficult to visualize bands less than 100 base pairs on these gels). By making the polymorphisms further apart, a single ASIP reaction can be performed that produces products that are long enough (351 base pairs and 366 base pairs) to be visualized on agarose gels. This is demonstrated by the fact that Li *et al.* circularizes to make the polymorphisms further apart in one direction, followed by inverse PCR that amplifies products of 351 base pairs and 366 base pairs which are much longer than the 30-43 base pairs that separates C/T and G/T. If Li *et al.* had intended to bring polymorphisms closer together, they would have done so by circularizing to bring the polymorphisms closer together for a single PCR primer to be designed, and they would have then used a forward, not an inverse PCR, off of the circularized template. In contrast, Applicants' method is designed to haplotype polymorphisms that are distantly located in *genomic* DNA by bringing the polymorphisms into closer proximity via circularization.

10. In greater detail, Li *et al.* faced several problems when haplotyping the MN blood group system (the subject of their article). First, the polymorphisms Li *et al.* utilized to type the M^G, M^T, and N alleles of the GPA gene also occurred in the GPB and GPE genes, which are over 95% homologous with the GPA gene. Li *et al.* solved this by carrying out GPA-specific PCR. Li *et al.*, page 358, column 2. Second, the M^G, M^T and N alleles cannot be typed by the single allele-specific PCR amplification technique. Li *et al.*, page 358, column 1. Li *et al.* explains the problem as follows.

> Although primers specific to M^G and N alleles can be designed, the M^T- (and N-) specific nucleotide T in intron 1 is located *too far* (30-40 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T-specific primer.

Li *et al.*, page 358, columns 1-2 (emphasis added). The inability to design a single M^T-specific primer can be overcome by allele-specific nested PCR after GPA-specific PCR. Li *et al.*, page 361, column 1. However, Li *et al.* chose to use circularization and one round of ASIP, which allows for a *single* procedure using allele-specific primers. Li *et al.*, sentence spanning pages 360-1. Li *et al.* correctly notes on page 361 that multiple allele-specific PCRs (nested necessarily refers to multiple) can be performed for haplotpe analysis after GPA-specific amplification, so the statement on page 361 refers back to the problem on page 358, in that "AISP [a single procedure]...can be applied to haplotyping polymorphisms separated by a distance that is too far to be amplified by PCR." It is my scientific opinion that this sentence refers to the fact that the polymorphisms are separated by a distance too great for a *single* primer to be designed that could anneal to a region that contains multiple polymorphisms (in intron1 and exon2 here) for a *single* allele-specific PCR procedure (and thus requiring either nested/multiple PCRs or Li *et al.*'s single AISP method). PCR here means a single PCR reaction, rather than if they had said nested PCR, which requires multiple PCR reactions. Thus, the problem of distance Li *et al.* refers to is polymorphisms being too far apart to design a primer for a single PCR procedure, and not a problem of polymorphisms being too far apart in genomic DNA for conventional haplotype analysis, as Li *et al.* notes that these polymorphisms can be haplotyped by RFLP and SSCP. Li *et al.* recognizes the following three problems of distance:

1. Too far for primer design for a single PCR reaction
2. Too close for visualizing short products of allele-specific PCRs on a gel
3. Having to perform multiple nested PCRs to overcome these 2 distance problems

Li *et al.* circumvents the need for multiple nested PCRs by circularizing to make the polymorphisms farther apart, followed by a single ASIP procedure that does not require a primer to anneal to a region containing multiple polymorphisms and produces products that are long enough to clearly see on a gel.

11. It is my scientific opinion that Li *et al.* performs circularization and ASIP to circumvent the need for allele-specific nested PCRs from genomic DNA, not to bring polymorphisms closer together. In other words, the last statement by Li *et al.* means that ASIP should be used in place of PCR, not combined with PCR.

12. It is my scientific opinion that the combination of the methods of Li *et al.* and Michalatos-Beloin *et al.* does not produce our claimed method. If one were to generate hemizygous templates with Michalatos-Beloin, then perform the circularization and ASIP of Li, it would not generate a shorter fragment with the polymorphisms closer together because (1) the circularization step does not bring the polymorphisms closer and (2) Li *et al.'s* PCR primers for the ASIP are in opposite orientation from ours (compare Li, Figure 1 with Figure 1 of our application). This in itself is conceptually *different* from our method, as we do not perform inverse PCR as exemplified by Li *et al.* from circularized templates. It is impossible to generate the fragments for haplotype analysis that our method does by using inverse PCR off of circular molecules.

13. In summary, the combination of Li *et al.* with Michalatos-Beloin *et al.* results in allele-specific amplification producing hemizygous target, followed by circularization and allele-specific inverse PCR, which simply regenerates the same or similar linear fragment that was first amplified by the Michalatos-Beloin isolation method. Notably, Li's method of circularization does not bring the nucleotide polymorphisms closer together. Patel *et al.* cannot be combined

with these other references to produce our method because Patel *et al.* teaches circularization of genomic DNA before isolation of the target (unlike Michalatos-Beloin *et al.* and Li *et al.*, which both isolate the target first).

14. For the above reasons, based on my education and scientific experience, I believe that one working in this field would not arrive at our method given the references by Li *et al.*, Patel *et al.*, and Michalatos-Beloin *et al.* Based on my education and scientific experience, I further believe that one working in this field would not be motivated to combine or modify these references based on the references themselves or the state-of-art.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Oliver Gene McDonald — Date: 3-11-04

William Edward Evans — Date: 3-17-04